Functional equivalency between *Otx2* and *Otx1* in development of the rostral head

Yoko Suda¹*, Jun Nakabayashi¹,2*, Isao Matsuo¹ and Shinichi Aizawa¹‡

¹Department of Morphogenesis, Institute of Molecular Embryology and Genetics, ²Department of Psychiatry, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto-860, Japan

*The first two authors contributed equally
‡Author for correspondence (e-mail: saizawa@gpo.kumamoto-u.ac.jp)

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SUMMARY

Mice have two *Otx* genes, *Otx1* and *Otx2*. Prior to gastrulation, *Otx2* is expressed in the epiblast and visceral endoderm. As the primitive streak forms, *Otx2* expression is restricted to the anterior parts of all three germ layers. *Otx1* expression begins at the 1 to 3 somite stage in the anterior neuroectoderm. *Otx2* is also expressed in cephalic mesenchyme. *Otx2* homozygous mutants fail to develop structures anterior to rhombomere 3 (r3), and *Otx2* heterozygotes exhibit craniofacial defects. *Otx1* homozygous mutants do not show apparent defects in early brain development. In *Otx1* and *Otx2* double heterozygotes, rostral neuroectoderm is induced normally, but development of the mes/diencephalic domain is impaired starting at around the 3 to 6 somite stage, suggesting cooperative interactions between the two genes in brain regionalization. To determine whether *Otx1* and *Otx2* genes are functionally equivalent, we generated knock-in mice in which *Otx2* was replaced by *Otx1*. In homozygous mutants, gastrulation occurred normally, and rostral neuroectoderm was induced at 7.5 days postcoitus (7.5 dpc), but the rostral brain failed to develop. Anterior structures such as eyes and the anterior neural ridge were lost by 8.5 dpc, but the isthmus and r1 and r2 were formed. In regionalization of the rostral neuroectoderm, the cooperative interaction of *Otx2* with *Otx1* revealed by the phenotype of *Otx2* and *Otx1* double heterozygotes was substitutable by *Otx1*. The otocephalic phenotype indicative of *Otx2* haploinsufficiency was also largely restored by knocked-in *Otx1*. Thus most *Otx2* functions were replaceable by *Otx1*, but the requirement for *Otx2* in the anterior neuroectoderm prior to onset of *Otx1* expression was not. These data indicate that *Otx2* may have evolved new functions required for establishment of anterior neuroectoderm that *Otx1* cannot perform.

Key words: Head organizer, *Otx2*, *Otx1*, Visceral endoderm, Rostral brain induction, Brain regionalization, Cephalic neural crest cells, Isthmus, Knock-in, Homeobox, Mouse

INTRODUCTION

The vertebrate head is a unique structure; no comparable structure is seen in cephalochordates or urochordates (Balfour, 1881; Goodrich, 1930; DeBeer, 1937; Northcutt and Gans, 1983; Butler and Hoods, 1996; Janvier, 1996; Niewenhuys, 1977). How head development and regionalization occur is an area of intense investigation in developmental biology. Both processes start at gastrulation, and Spemann and Mangold distinguished the head organizer, which induces the anterior part of the body, from the trunk organizer (1924). In mice, the visceral endoderm is now being investigated as the site of head organizer activity (Thomas and Beddington, 1996; Varlet et al., 1997; Belo et al., 1997; Beddington and Robertson, 1998; Biben et al., 1998; Shwalot et al., 1998; Thomas et al., 1998; Waldrip et al., 1998).

As gastrulation proceeds the region of the rostral head is determined throughout the three germ layers. Roles for prechordal mesendoderm in patterning of the rostral brain have been suggested (Nieuwkoop, 1950; Toivonen and Saxen, 1968; Ang et al., 1994; Ang and Rossant, 1994; Weinstein et al., 1994; Chiang et al., 1996; Foley et al., 1997; Dale et al., 1997). The isthmus and anterior neural ridge have been proposed as centers involved in anterior/posterior patterning in the midbrain and forebrain, respectively (Marin and Puelles, 1994; Crossley and Martin, 1995; Crossley et al., 1996; Joyner et al., 1996; Shimamura and Rubenstein, 1997; Houart et al., 1998). Regionalization in the rostral brain generates telencephalon, diencephalon, mesencephalon and the isthmus, followed by further subdivisions (Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Cephalic neural crest cells also play essential roles in head development; they form structures such as anterior cranium and cranial nerves (Northcutt and Gans, 1983; Matsuo et al., 1995). Recently, several genes have been proposed to play critical roles in the processes of head development. *Otx2*, a *bicoid*-class homeobox gene, is one such gene.

*Otx2* is first expressed in the epiblast (Simeone et al.,
1993). Before gastrulation it is also expressed in the anterior visceral endoderm (Acampora et al., 1995). Otx2 homozygous mutants fail to develop structures anterior to rhombomere 3 (r3), as do Lim1 mutants (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996). Subsequently, Otx2 is expressed in anterior parts of all three germ layers. Otx1 expression follows in the anterior neuroectoderm, and the caudal limit of expressions of both Otx genes in the neuroectoderm coincides with the boundary between the midbrain and hindbrain. Emx2 and Emx1 are then expressed in a more limited region. The expression patterns of Otx and Emx genes suggest a combinatorial code for rostral brain development, analogous to the Hox code for hindbrain development (Simeone et al., 1992). Cooperative functions between Otx1 and Otx2 genes in the formation of mesencephalon and caudal diencephalon were demonstrated by Otx1 and Otx2 double mutants (Suda et al., 1996, 1997; Acampora et al., 1997). The Otx2 gene also cooperates with the Emx2 gene in development of the telencephalon and anterior diencephalon (Yoshida et al., 1997; our unpublished result). In addition, Otx2 is expressed in cephalic neural crest cells (Kimura et al., 1997), and Otx2 heterozygotes exhibit otoccephaly due to defects in these cells (Matsuo et al., 1995).

All gnathostomes have two lineages of Otx cognates: Otx1 and Otx2. The Otx2 cognates are highly conserved, while Otx1 cognates are relatively diverged in gnathostomes. Xenopus and zebrafish, in which polyploidization took place independently, Otx2 cognates are highly conserved, while Otx1 cognates in tetrapods are relatively diverged in gnathostomes (Freund et al., 1997; Furukawa et al., 1997; Ueki et al., 1998). In an agnathous vertebrate, the freshwater lamprey, we have also identified two Otx cognates, LjOtxA and LjOtxB (Ueki et al., 1998). In contrast, Amphioxus and ascidians, sister groups of vertebrates, appear to have only one Otx gene (Wada et al., 1996; Williams and Holland, 1998). There are structural differences between vertebrate and invertebrate Otx cognates (Freund et al., 1997; Furukawa et al., 1997; Ueki et al., 1998). It is tempting to speculate that Otx gene duplication contributed to, or permitted, the evolution of the rostral brain in vertebrates and that each gene recruited different downstream targets. In mice, however, the head develops apparently normally with only one Otx gene, Otx2: Otx1 null mutants exhibit brain defects only later in neurogenesis (Acampora et al., 1996; Suda et al., 1996, 1997). In lamprey, LjOtxB is not expressed in rostral neuroectoderm, and rostral brain expresses only one gene, LjOtxA (Ueki et al., 1998). Thus, it is probable that the vertebrate brain evolved with only one Otx gene in ancestral agnatha and that Otx gene duplication took place independently in lineages of the extant agnatha and gnathostomes.

In the gnathostome lineage, it is Otx2 cognates in tetrapods but Otx1 in zebrafish that are first expressed in early gastrulation and during early anterior neurulation (Simeone et al., 1993; Li et al., 1994; Mori et al., 1994; Bally-Cuif et al., 1995; Mercier et al., 1995; Pannese et al., 1995; Kablar et al., 1996; Ueki et al., 1998). It was recently reported that human OTX1 and OTX2 can substitute for functions of orthodenticile (otd), the Drosofila orthologue of Otx (Finkelstein et al., 1990), in the fruit fly (Leuzinger et al., 1998; Nagao et al., 1998). Likewise, otd can substitute for Otx1 in mice (Acampora et al., 1998). To address the question of whether Otx1 is functionally equivalent to Otx2, we generated mice in which Otx2 was replaced with Otx1. Analysis of the knock-in mice showed that most Otx2 functions are replaceable with those of Otx1, but that those in the anterior neuroectoderm prior to the onset of the Otx1 expression are not.

MATERIALS AND METHODS

Construction of targeting vectors

Mouse Otx1 and Otx2 cDNAs were isolated from a mouse 11 dpc cDNA library (Clontech); the nucleotide sequences were confirmed by standard sequencing methods. To construct the replacement vector, the EcoRI-BamHI fragment of pKJ2 (Boer et al., 1990) containing the neo cassette (the neo resistance gene driven by the phosphoglycerate kinase 1 (PGK1) promoter and lacking a polyadenylation signal) was inserted into BamHI and HindIII sites into two loxP sites of pBS246 (Gibco BRL) by blunt end ligation. A fusion gene consisting of the 5′ non-coding region of Otx2 cDNA, the coding region of Otx1 cDNA, the 3′ non-coding region of Otx2 cDNA, the SV40 t splice site and the SV40 polyadenylation signal was constructed (Fig. 1B) and flanked by SmaI and EcoRV sites at the 5′ and 3′ ends, respectively. The Sma/EcoRV fragment was fused with the EcoRV/Scl fragment of the neo cassette flanked with loxP. The resulting fusion product was inserted between two SmaI sites that flank the translation initiation codon of Otx2, one located 213 bp upstream and the other 21 bp downstream of the ATG. The length of the homologous region was 6.7 kb and 4.1 kb at the 5′ and 3′ sides of the insert, respectively (Fig. 1A). The targeting vector contained a diphtheria toxin A fragment (DT-A) gene driven by the MCM promoter for positive selection as described by Yagi et al. (1993b). A targeting vector for the lacZ knock-in was constructed using the same strategy. Details of the construction of the targeting vectors are available upon request.

Generation of knock-in mice

TT2 ES cells (Yagi et al., 1993a) were cultured, electroporated with the Sall-linearized targeting vector and selected in G418 as described by Nada et al. (1993); Yagi et al. (1993b). Homologous recombinants were obtained at a frequency of 11/191 and of 5/139 G418 resistant clones for Otx1 and lacZ knock-ins, respectively.

Chimeric mice were obtained by injecting homologously recombinated TT2 cells into 8 cell embryos as described by Yagi et al. (1993a). Male chimeras were mated with C57BL/6 females. The resulting zygotes were injected with the Cre gene driven by the chicken β-actin promoter to delete the loxP-flanked neo cassette, as described by Araki et al. (1995). Otx1 and Otx2 mutants were generated previously (Matsuo et al., 1995; Suda et al., 1996). The genetic background of mice used in the present study is described in the Results. The mice were housed in environmentally controlled rooms of the Laboratory Animal Research Center of Kumamoto University School of Medicine under University guidelines for animal and recombinant DNA experiments.

Genotyping

Genotypes of newborn mice and embryos were routinely determined by PCR (polymerase chain reaction) analyses and confirmed, when necessary, by Southern blots of genomic DNAs prepared from tails or yolk sacs. In the PCR analyses, the primers and the length of the resulting zygotes were injected with the primers and the length of the PCR products were as follows: wild-type Otx2 allele (305 bp) with primer OX24 (5′-TAGTTATGAGAATGAGGAAGCTGC-3′) and primer p104 (5′-ATACATCGGAAACCAGGTTGAGTGTGC-3′); Otx1 knock-in Otx2 allele (728 bp) with primer SV2 (5′-GCCTTTAGAATGAACTACGCG-3′) and primer p104; lacZ knock-in Otx2 allele (600 bp) with primer N3 (5′-GCTTGTTCGCGCAATGATCGTGGGAAAAT-3′) and primer p105 (5′-AATGCTCTGTGCGACTCGGACGTTTGGTAGG-3′). The Otx2 and
In situ hybridization

Embryos were dissected in PBS and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Embryos were gradually dehydrated in methanol/PBT (PBS-0.1% Tween-20) up to 100% methanol and stored at −20°C. The protocol for whole mount in situ hybridization of embryos was as described by Wilkinson (1993), using single-stranded digoxigenin-UTP labeled RNA probes (Boehringer Mannheim). The probes used were those described for Wnt1 (McMahon and Bradley, 1990), En1 (Davis and Joyner, 1988), Pax2 (Dressler et al., 1990), Fgf8 (Crossley and Martin, 1995), Emx2 (Yoshida et al., 1997), Otx1 and Otx2 (Matsuo et al., 1995), Krox20 (Wilkinson et al., 1989), Six3 (Oliver et al., 1995), Hex1/Rpx (Hermesz et al., 1996), Fkh2 (Kaestner et al., 1995), HNF3β (Sasaki and Hogan, 1993), Brachyury (Herrmann, 1991) and cer-1 (Belo et al., 1997).

RT-PCR analysis

RT (reverse transcription)-PCR analyses were performed with total RNAs isolated from 7.5 dpc embryos and reverse-transcribed with oligo(dT)17 (Ilic et al., 1995). The primers used for the Otx1 expression were p1 (5'-ATGATGTCTTACCTAAACAACCC) from the first exon and p2 (5'-TGAGCGCGTGTTGAGTGCGCTC) from the second exon. Those for Otx2 expression were RT1 (5'-TCTTATCTAAAGACACCCGCTTACGAGTCT) from the first exon and OX2A (5'-GACACTCGGATTGCTGGAATTTTA) from the second exon.

β-Gal staining

Embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in PBS for 10 minutes, followed by three washes with PBS for 10 minutes at room temperature. Staining was carried out overnight at 37°C in PBS containing 1 mg/ml X-Gal, 20 mM K4Fe(CN)6, 20 mM K3Fe(CN)6, 2 mM MgCl2, and 0.02% NP-40. Stained embryos were washed twice with PBS and immediately stored in 10% formaldehyde.

Western blot analysis

A rabbit anti-mouse Otx1 peptide (GGSYGQQYPAPSSSY) polyclonal serum was generated by standard procedures (Research Genetics) and purified twice on a protein A sepharose (Amersham Pharmacia) and on an affinity column of Otx1 peptide immobilized and purified twice on a protein A sepharose (Amersham Pharmacia) and on an affinity column of Otx1 peptide immobilized (Oyanagi et al., 1997). For Western blot analysis, stained embryos were washed twice with PBS and immediately stored.

RESULTS

Otx1 knock-in into Otx2 locus

Fig. 1A shows the targeting vector used to knock Otx1 into the Otx2 locus. Since the transcriptional regulatory elements of the Otx2 gene are not yet fully characterized (Kimura et al., 1997), Otx1 cDNA was inserted at the translation start site of the Otx2 gene to minimize deletion of Otx2 sequences (see Materials and Methods). The Otx1 cDNA contained two polyadenylation signals, one derived from Otx2 and the other from SV40 early genes, followed by the neomycin resistance gene driven by the PGK1 promoter and lacking a polyadenylation signal (neo cassette). To avoid potential interference with Otx2 transcriptional regulatory elements by the PGK1 promoter, the neo cassette was flanked with loxP sites for deletion with Cre recombinase. Since it was possible that differences in non-coding sequences in Otx2 and Otx1 cDNAs might affect RNA stability or translational regulation, all non-coding sequences of Otx1 cDNA were replaced with those from Otx2 cDNA, as shown in Fig. 1B.

Homologous recombinants were obtained using TT2 ES cells derived from an F1 embryo from C57BL/6xCBA mice. To delete the neo cassette, yzogotes obtained from crosses of male chimeras with C57BL/6 females were injected with the Cre gene under control of the chicken β-actin promoter (Araki et al., 1995) and then allowed to develop in foster mothers to generate F1 offspring. F1 mice in which the deletion of the neo cassette was confirmed (Fig. 1C) were crossed with C57BL/6 to analyze the heterozygous phenotype. The homozgyous phenotype was examined in the F3 generation following crosses among F2 heterozygous knock-in mice. Two independent knock-in mouse strains were established from two independent homologous recombinant ES lines. No difference was observed between them in regard to the phenotypes described below. As controls, previously established Otx2 null mutants (Matsuo et al., 1995) were examined in the same generations by the same crosses. The F2 heterozygous and F3 homozygous phenotypes of the null mutation were identical to those previously reported. The mutated loci are designated below as Otx2+/− and Otx2−/− for the respective heterozygous and homozygous null mutation and Otx2−/+;otx1−/− and Otx2−/+;otx1−/− for the respective heterozygous and homozygous knock-in mutations.

To determine whether the knocked-in Otx1 gene is properly expressed in place of the Otx2 gene, we first examined β-Gal expression in mice in which the Otx2 gene was replaced with the lacZ gene using the same strategy. The mice showed β-Gal expression in the visceral endoderm, anterior mesendoderm, the anterior neuroectoderm to the caudal boundary at the midbrain/hindbrain junction, cephalic mesenchyme and other regions known to express Otx2, by in situ hybridization (Fig. 6P; Acampora et al., 1995). Second, we determined the mRNA level in heterozygous (Otx2+/otx1−) and homozygous knock-in (Otx2−/+;otx1−/−) embryos by quantitative RT-PCR at 7.5 dpc when normally Otx2, but not Otx1, is expressed (Fig. 2A, lane 2). In the Otx2−/+;otx1−/− embryos, Otx1 was expressed instead of
Otx2 as predicted (lane 3). In addition, analysis of the Otx2<sup>+/otx1</sup> embryos showed that Otx1 and Otx2 were expressed at approximately the same levels (lane 4). Third, we conducted in situ hybridization analyses of Otx1 and Otx2 expressions in Otx2<sup>+/otx1</sup> embryos at the same stage. The Otx1-positive region was almost identical in pattern to the Otx2-positive region (Fig. 2B). In situ analysis of Otx2<sup>otx2otx1</sup> embryos also verified the replacement of Otx2 expression by Otx1 in knock-in embryos (Fig. 6B). Finally, we analyzed the Otx1 protein product in 8.0 dpc knock-in embryos, the stage at which Otx2 is expressed mainly in the anterior neuroectoderm of wild-type Otx1 embryos, the stage at which defects in the eyes and lower jaw, but similar expression in the visceral endoderm and definitive mesendoderm is residual, and Otx1 expression in the neuroectoderm is minimal (Fig. 6P-R, Fig. 9). The knocked-in Otx1 was expressed as a 40 kDa protein product as predicted (Fig. 2C).

**Otx2 heterozygous phenotype**

Otx2 heterozygous mutants (Otx2<sup>+/−</sup>) exhibit craniofacial malformations (Matsuo et al., 1995), a phenotype observed in a C57BL/6 genetic background but suppressed in a CBA background. TT2 ES cells have the F<sub>1</sub> genetic background of CBA and C57BL/6 mice (Yagi et al., 1993a). Thus, it was necessary to determine which Otx2 allele, C57BL/6 or CBA, was targeted in the knock-in and the null mutations. To do so we found a polymorphic site located about 1.5 kb upstream of the translation start site that discriminates between the CBA and C57BL/6 Otx2 loci (Oyanagi et al., 1997). The targeting vectors for the null and knock-in mutations were both made using CBA Otx2 genomic DNA. An example of the analysis is shown in Fig. 3A. When the F<sub>2</sub> Otx2<sup>+/-</sup> or Otx2<sup>+/otx1</sup> mice were mated with C57BL/6 mice, all the wild-type offspring bore only the C57BL/6-derived Otx2 allele, whereas all the heterozygotes bore both C57BL/6- and CBA-derived alleles. In turn, when the F<sub>2</sub> Otx2<sup>+/-</sup> or Otx2<sup>+/otx1</sup> mice were mated with CBA mice, all the wild-type offspring bore both C57BL/6- and CBA-derived alleles, while all the heterozygotes bore only the CBA-derived Otx2 allele. This data confirms that in both the null and the knock-in mutations, recombination took place in the CBA Otx2 allele of TT2 ES cells. Thus, any potential difference between the heterozygous phenotype observed in the null mutation and the knock-in mutation is not attributable to a difference in the origin of targeted Otx2 allele.

The Otx2<sup>+/−</sup> phenotype in a C57BL/6 genetic background is variable (Matsuo et al., 1995). Acephaly was observed in about 3% of the Otx2<sup>+/−</sup> mice but never seen in Otx2<sup>2+otx1</sup> mice (Fig. 3B,C). About 53% of the Otx2<sup>+/−</sup> mice had defects in the eyes and lower jaw, but similar
747 Otx2 and Otx1 in head development were seen in only 3% of the Otx2+/otx1 mice. The proportion of Otx2+/- mice showing defects only in eyes or only in lower jaw was approximately 17% and 6%, respectively; in Otx2+otx1 mice the percentages were 3% and 4%, respectively. In the Otx2+/- mice, only 16% showed no apparent defects, yet 86% of Otx2+otx1 mice were apparently

normal. Craniofacial defects seen in Otx2+/- mice (i.e., in the trabecular components of cranium, the ophthalmic branch of the trigeminal nerve and in mesencephalic trigeminal neurons (Matsuo et al., 1995)) were also largely restored in Otx2+otx1 mice (data not shown). We previously identified the craniofacial anomalies of Otx2+/- mice as defects in cephalic neural crest cells (Matsuo et al., 1995; Kimura et al., 1997). Therefore we suggest that Otx1 can substitute for Otx2 function in these cells.
Homozygous knock-in phenotype

*Otx2*<sup>−/−</sup> embryos did not develop beyond 9.5 dpc (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), but *Otx2<sup>otx1/otx1</sup>* mice were live-born, although they did not survive. Before birth, at 18.5 dpc, 23 out of 56 offspring from crosses among heterozygotes were homozygous knock-in mutants. These *Otx2<sup>otx1/otx1</sup>* embryos, however, suffered from severe otocephaly. Some embryos lacked the rostral head, while most developed some rostral structures (Fig. 4A-D). Otic vesicles, the medulla oblongata, the trigeminal ganglion, choroid plexus in the fourth cerebral ventricle, the trachea, the entrance to the esophagus, the hyoid bone, Meckel’s cartilage and the basisphenoid bone were present in all prenatal knock-in embryos. Many *Otx2<sup>otx1/otx1</sup>* embryos developed a tongue, oropharynx and mandible (though small) with teeth and lower lip; the maxillary bone was never observed. The cerebellum, pons and some structures anterior to both were also formed in some embryos, but the forebrain, hypophysis and most anterior structures such as eyes or olfactory bulbs were never formed. Thus, knocked-in *Otx1* can rescue some but not all *Otx2* functions in the rostral head.

*Otx2<sup>otx1/otx1</sup>* embryos were next examined at 11.5 dpc (Fig. 4E,F). Otic vesicles and the trigeminal ganglion were present. The isthmic constriction was obscure in some embryos but observable in most, and more caudal structures were apparently normal. An infundibulum-like structure was present, as was the cephalic flexure, and some anterior structures were observed. Eyes were never formed.

**Early defects in the rostral brain**

To determine the affected regions more precisely, an analysis was undertaken with molecular markers at 8.5 dpc. Development was generally retarded in homozygous knock-in embryos. The *Otx2<sup>−/−</sup>* embryos never develop regions anterior to r3 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). In homozygous knock-in embryos, *Krox20* expression in r3 and r5 and *Fgf8* expression in the isthmus were normal (Fig. 5A-D); *Pax2* expression in the isthmus was present though weak compared to wild type. *Fgf8* was normally expressed in a transverse band in the 9.5 dpc isthmus (data not shown). In wild-type embryos *Otx1* and *Otx2* are expressed in forebrain and midbrain with the caudal boundary of both at the junction with the isthmus. *Otx2<sup>otx1/otx1</sup>* embryos had *Otx1*-positive region, although the region was greatly reduced (Fig. 5G,H). No *Otx2*...
expression was detected as expected (data not shown). In wild-type embryos En1 is expressed from r1 to the midbrain, and Wnt1 is expressed from the isthmus to the caudal diencephalon. In the Otx2\textsuperscript{otx1/otx1} embryos, both En1 and Wnt1 expressions were markedly reduced (Fig. 5I-L). It is noteworthy that almost no morphological structure was observed anterior to the Wnt1-positive region (Fig. 5K,L). Normally Emx2 is expressed in the telencephalon and anterior diencephalon; in homozygous knock-in embryos its expression was barely detectable (Fig. 5A,B). In wild-type embryos Six3 expression is seen in the anterior ridge of the forebrain. In the Otx2\textsuperscript{otx1/otx1} embryos, this expression was absent (Fig. 5M,N), but expression in the ventral diencephalon, which may correspond to the future infundibulum, was retained. Hesx1 was expressed weakly in the future Rathke’s pouch but not seen in the anterior neuroectoderm (Fig. 5O,P). Fgf8 expression was also observed in the ventral diencephalon but not in the anterior neural ridge (Fig. 5C.D). Overall, marker analysis showed normal development of the isthmus and r1/2, defects in the midbrain and loss of dorsal forebrain. Variation in these markers was minimal in 8.5 dpc Otx2\textsuperscript{otx1/otx1} embryos, and alterations in expression of markers compared to wild-type patterns were predictive of the 11.5 dpc phenotype.

**Induction of rostral neuroectoderm**

Defects in homozygous knock-in embryos were further examined at 7.5-7.75 dpc, the initial stage of rostral neuroectoderm development. Otx2 (Simeone et al., 1993), Six3 (Oliver et al., 1995), Fkh2 (Kaestner et al., 1995), Hesx1/Rpx (Thomas and Beddington, 1996; Hermesz et al., 1996) and Pax2 (Rowitch and McMahon, 1995) are the earliest markers of rostral neuroectoderm. Otx2\textsuperscript{−/−} embryos never expressed these markers at this stage (Fig. 6C,F,I,L,O); Pax2 expression was not observed in our 7.5-8.5 dpc Otx2\textsuperscript{−/−} embryos (Fig. 6O) despite the recent observation by Rhinn et al. (1998). In the Otx2\textsuperscript{otx1/otx1} embryos, knocked-in Otx1 expression was indistinguishable from Otx2 expression seen in wild-type embryos (Fig. 5).

**Fig. 5.** Expression of anterior neural markers in 8.5 dpc rostral brain. (A,C,E,G,I,K,M,O) Wild-type and (B,D,F,H,J,L,N,P) Otx2\textsuperscript{otx1/otx1}. (A,B) Krox20 and Emx2, (C,D) Fgf8, (E,F) Pax2, (G,H) Otx1, (I,J) En1, (K,L) Wnt1, (M,N) Six3 and (O,P) Hesx1 expressions. In Otx2\textsuperscript{−/−} embryos, the Krox20-positive r3 is at the most anterior end, and the other genes are never expressed (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Arrows in (A,B) show Emx2 expression in forebrain that is faint in Otx2\textsuperscript{otx1/otx1} embryos, and in (C-F) expression in isthmus. Open triangles in (C,D,M,N) indicate expression in ventral diencephalon, which corresponds to the future infundibulum, and in (O,P) Rathke’s pouch; arrowheads in (C,M,O) indicate Fgf8, Six3 and Hesx1 expression in the anterior neural ridge or neuroectoderm that is absent in Otx2\textsuperscript{otx1/otx1} embryos (D,N,P). Asterisks in C,D indicate Fgf8 expression in the foregut, and in E Pax2 expression in the optic stalk. Scale bars, 150 μm.
6A,B). Six3, Fkh2 and Pax2 expression patterns were also normal in homozygous knock-in embryos (Fig. 6D,E,G,H). The region anterior to Pax2 expression was, however, somewhat reduced (Fig. 6M,N), and expression of all these markers decreased by 8.0 dpc (data not shown). Hesx1 expression was faint even in the 7.5 dpc Otx2\textsuperscript{+\textasciitilde/\textasciitilde}embryos (Fig. 6K). We also compared β-Gal expression at 7.75 dpc in Otx2\textsuperscript{+\textasciitilde/lacZ}and Otx2\textsuperscript{otx1/lacZ}embryos (Fig. 6P-R). In Otx2\textsuperscript{otx1/lacZ}embryos at this stage, β-Gal expression was found mainly in anterior neuroectoderm, and expression in anterior definitive mesendoderm was residual. In Otx2\textsuperscript{otx1/lacZ}embryos at this stage β-Gal expression was found in anterior mesendoderm and induced in anterior neuroectoderm, though the level was lower than that seen in Otx2\textsuperscript{+\textasciitilde/lacZ}embryos. The Otx1 dosage effect should be kept in mind when comparing levels of β-Gal expressions (Fig. 6P-R) with those of Otx2 and Otx1 mRNA (Fig. 6A,B).

About half of the Otx2\textsuperscript{+\textasciitilde/c}\textasciitilde} embryos (11/20) undergo abnormal gastrulation, exhibiting a characteristic constriction at the junction of the extraembryonic and embryonic ectoderm at early to mid-streak stage (Fig. 7C). Similar abnormalities were also seen in Lim1 and HNF3β mutants (Ang and Rossant, 1994; Weinstein et al., 1994; Shawlot and Behringer, 1995). Such a phenotype was never observed in Otx2\textsuperscript{otx1/otx1} embryos (0/20) (Fig. 7B), which also expressed Brachyury normally (Fig. 7D-F). In the Otx2\textsuperscript{+\textasciitilde/c}\textasciitilde} embryos, HNF3β-positive anterior mesendoderm was not formed, but Otx2\textsuperscript{otx1/otx1}embryos developed the mesendoderm (Fig. 7G-I). In wild-type embryos, cer-l is expressed in anterior mesendoderm to the embryonic/extraembryonic junction (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998) (Fig. 7J). In Otx2\textsuperscript{+\textasciitilde/c}\textasciitilde}embryos cer-l expression either did not extend to the junction (Fig. 7L) or frequently remained in the distal tip of the mesendoderm (Biben et al., 1998); however, cer-l was expressed normally in the anterior mesendoderm of Otx2\textsuperscript{otx1/otx1}embryos (Fig. 7K).

**Cooperative function of Otx2 and Otx1 in development of mesencephalon and diencephalon**

Otx1 expression begins at 8.0 dpc, around the 1-3 somite stage (Simeone et al., 1992; Suda et al., 1997). Otx2 function in the neuroectoderm after this stage is apparent from the phenotype of Otx2 and Otx1 double heterozygous mutants (Otx1\textsuperscript{+\textasciitilde}/Otx2\textsuperscript{+\textasciitilde}): these mice show marked defects in mesencephalon and caudal diencephalon, while defects are not seen in either Otx1\textsuperscript{+\textasciitilde}/ or Otx2\textsuperscript{+\textasciitilde}/mutants (Suda et al., 1996, 1997). The defects observed in the double heterozygotes take place at the time of brain regionalization occurring around the 3 to 6 somite stage. Functional equivalency between Otx1 and Otx2 at this stage cannot be deduced from the Otx2\textsuperscript{otx1/otx1}phenotype, since

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**Fig. 6.** Expression of anterior neural markers at 7.5-7.75 dpc. (A,D,G,J,M) Wild type, (B,E,H,K) Otx2\textsuperscript{otx1/otx1}, (C,F,L,O) Otx2\textsuperscript{+\textasciitilde}, (P) Otx2\textsuperscript{+\textasciitilde/lacZ}and (Q,R) Otx2\textsuperscript{otx1/lacZ}, (A,C) Otx2, (B) Otx1, (D-F) Six3, (G-I) Fkh2, (J-L) Hesx1, (M-O) Pax2 and (P-R) β-Gal expressions. J-L show ventral views and others are lateral views. In Otx2\textsuperscript{otx1/otx1}embryos, Otx2 expression was not seen (Fig. 2A, lane 3), nor is endogenous Otx1 expressed at this stage (Fig. 2A, lane 2). Thus Otx1 expression in the Otx2\textsuperscript{otx1/otx1}embryo (B) represents that of the knocked-in Otx1. The embryo shown in Q in which β-Gal is expressed in anterior mesendoderm is less advanced than the embryo in R where β-Gal expression is already initiated in anterior neuroectoderm. Scale bars, 100 μm.
defects were seen in the anterior neuroectoderm of Otx2<sup>+/+/o</sup> mutants before the onset of Otx1 expression. Therefore to examine whether Otx1 can substitute for Otx2 function at this stage Otx1<sup>+/+/o</sup> double heterozygotes were analyzed.

In 10.5 dpc Otx1<sup>+/+/o</sup> embryos, the region between the otic vesicles and the isthmic constriction was enlarged (Fig. 8A,B), whereas between the constriction and the sulcus telodiencephalicus was shortened (Fig. 8A-D), as we previously reported (Suda et al., 1998): the telencephalon was smaller, the invagination of the sulcus telodiencephalicus was poor and the isthmic constriction was not distinct. In the majority of 10.5 dpc Otx1<sup>+/+/o</sup> embryos, the mesencephalon was somewhat smaller, the isthmic constriction was somewhat extended, and r1 was slightly expanded. However, the sulcus telodiencephalicus was present in a normal manner, and no defects were apparent in telencephalon or diencephalon. In prenatal Otx1<sup>+/+/o</sup> mutants, the midbrain, pretectum and dorsal thalamus were vestigial, while the anterior hindbrain, cerebellum and pons were expanded. The ventral thalamus, cerebral hemispheres and the hippocampal region were mildly reduced in size. In contrast, these defects were almost completely restored in most of the Otx1<sup>+/+/o</sup> mutants, although in some mutants the restoration was partial. Thus Otx2 function in the neuroectoderm after the onset of Otx1 expression may essentially be rescued by Otx1.

**DISCUSSION**

We have used a knock-in strategy to examine the functional equivalency of Otx1 and Otx2 genes. Our findings suggest that Otx1 can substitute for Otx2 function in the visceral endoderm, the anterior mesendoderm and cephalic neural crest cells. Otx1 can also replace Otx2 function in the anterior neuroectoderm after the onset of Otx1 expression. Otx1 cannot, however, substitute for Otx2 function in the neuroectoderm prior to the onset of Otx1 expression. The amino acid identity between mouse Otx1 and Otx2 is 98% in homeodomains, 81% in the N-terminal region and 44% in the C-terminal region (Simeone et al., 1993). A chronology of rostral brain development and mutant defects described in this paper are shown schematically in Fig. 9, along with spatiotemporal changes in Otx gene expression. Otx1 and Otx2 functions in later neurogenesis fall outside the scope of this study.

**Knock-in mutation**

Otx2<sup>+/+</sup> and Otx1<sup>+/+</sup>Otx2<sup>+/+</sup> defects were restored in a majority of Otx2<sup>+/++/+</sup> and Otx1<sup>+/+</sup>Otx2<sup>+/++/+</sup> mutants, respectively, but restoration was partial in some embryos. The prenatal Otx2<sup>+/++/+</sup> phenotype was also variable with possible secondary defects, although it was fairly consistent at earlier stages. Whether a knocked-in gene is expressed as precisely as the replaced gene is a critical question, and that issue is particularly relevant to defects seen in early anterior neuroectoderm, which were not rescued in Otx2<sup>+/++/+</sup> embryos. Our belief that the knocked-in gene is expressed precisely as the replaced gene is a critical question, and the issue is particularly relevant to defects seen in early anterior neuroectoderm, which were not rescued in Otx2<sup>+/++/+</sup> embryos. The prenatal Otx2<sup>+/++/+</sup> phenotype was also variable with possible secondary defects, although it was fairly consistent at earlier stages.

In Otx2<sup>+/lacZ</sup> embryos, however, β-Gal was not expressed in the same knock-in strategy, and that such regulation might be also required for expression of knocked-in Otx1 in early anterior neuroectoderm. We, however, consider this possibility unlikely for several reasons. First, all noncoding sequences of the knocked-in Otx1 cDNA construct were replaced with corresponding sequences of Otx2. Second, in
Otx2^{+/lacZ} and Otx2^{otx1/lacZ} embryos in which the lacZ gene was knocked-in, β-Gal was expressed in anterior neuroectoderm. Third, the rescue of Otx1^{+/+}/Otx2^{+/+} defects in Otx1^{+/+}/Otx2^{+/otx1} embryos indicates that knocked-in Otx1 is expressed in the neuroectoderm. Finally, western blot analysis of Otx1 protein in 8.0 dpc Otx2^{otx1/otx1} embryos suggests that the protein is produced in the anterior neuroectoderm, because at this stage in wild-type embryos, Otx2 is expressed mainly in anterior neuroectoderm and Otx1 is not expressed.

**Early Otx2 functions**

Otx2 mRNA is first expressed in the mouse epiblast (Simeone et al., 1993). A role in cell proliferation has been suggested (Ang et al., 1996), but Otx2 function is not yet determined. Prior to primitive streak formation Otx2 is also expressed in the visceral endoderm (Acampora et al., 1995). That expression localizes to the future anterior region of the embryo, as does expression of cer-l, Hexl, Lim1, nodal, Hex and HNF3β (Hermesz et al., 1996; Thomas and Beddington, 1996; Belo et al., 1997; Varlet et al., 1997; Beddington and Robertson, 1998; Biben et al., 1998; Shawlot et al., 1998; Thomas et al., 1998). Expression of these genes in the extraembryonic endoderm may play a role in head organizer activity, the loss of which leads to developmental failure of structures anterior to r3 in Otx2- and Lim1-deficient embryos (Beddington and Robertson, 1998; Rhinn et al., 1998). Otx2, Lim1 and HNF3β mutants all exhibit abnormal gastrulation (Ang and Rossant, 1994; Weinstein et al., 1994; Shawlot and Behringer, 1995). As primitive streak formation progresses, Otx2 expression in the epiblast is lost caudally and becomes confined to the anterior region through the three germ layers (Simeone et al., 1993; Acampora et al., 1995). In avians, Otx2 mRNA expression in the epiblast once disappears, becomes confined to Hensen’s node and then localizes to the anterior neuroectoderm (Bally-Cuif et al., 1995).

In Otx2^{otx1/otx1} embryos, gastrulation defects were not observed, and HNF3β-positive anterior mesendoderm was formed. These embryos expressed cer-l, a candidate rostral neuroectoderm inducer (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), normally. Expression of Six3, Fkh2 and Pax2, and knocked-in Otx1 and β-Gal suggest induction of anterior neuroectoderm in knock-in embryos. Thus we conclude that any early functions of Otx2 in the epiblast, in visceral endoderm and in anterior mesendoderm are substitutable with Otx1.

**Otx2 function in establishment of rostral neuroectoderm**

Morphologically, rostral brain develops as prosencephalic and mesencephalic vesicles (Puelpes et al., 1987). The caudal limit of Otx2 expression in anterior neuroectoderm, though initially obscure, becomes distinct in the mesencephalic vesicle; the Otx2-positive region generates the midbrain and the Otx2-negative region becomes the isthmus (Bally-Cuif and Wassef, 1995; Millet et al., 1996). Otx1 expression occurs around the time of this process at 8.0 dpc or the 1-3 somite stage (Simeone et al., 1993; Suda et al., 1997). Thus, the establishment of anterior neuroectoderm is initiated in the absence of the Otx1 expression.

Analysis using molecular markers at 7.5 to 8.5 dpc suggested that anterior neuroectoderm was induced but failed to develop in Otx2^{otx1/otx1} embryos. At 7.5 dpc Six3, Fkh2, Pax2 and knocked-in Otx1 were expressed fairly normally in anterior neuroectoderm, but their expression decreased by 8.0-

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**Fig. 8. Rescue of Otx1^{+/-}/Otx2^{+/-} defects in Otx1^{+/-}/Otx2^{+/otx1} mutants. (A,D,G) Wild type, (B,E,H) Otx1^{+/-}/Otx2^{+/-} and (C, F, I) Otx1^{+/-}/Otx2^{+/otx1}. (A-F) 10.5 dpc, (G,I) newborn and (H) 18.5 dpc. Otx1^{+/-}/Otx2^{+/-} mutants are not live-born (Suda et al., 1996, 1997). Arrows indicate sulcus telodiencephalicus, arrowheads isthmic constriction and open arrowheads lesser isthmic constriction in Otx1^{+/-}/Otx2^{+/-} mutants. Abbreviations: cb, cerebellum; di, diencephalon; dt, dorsal thalamus; ge, ganglionic eminence; hi, hippocampal region; ic, inferior colliculus; m, mammillary region; me, mesencephalon; mt, metencephalon; ob, olfactory bulb; ov, otic vesicle; p, pons; pt, pretectum; sc, superior colliculus; te, telencephalon; tm, tegmentum; vt, ventral thalamus. Scale bars, 500 μm.
8.5 dpc. At 7.5 dpc Hesx1 expression was barely detectable, and the region anterior to the Pax2-positive domain was somewhat reduced in size. Signals from the visceral endoderm and/or anterior mesendoderm may induce Otx2, Six3, Fkh2, Hesx1 and Pax2 expression independently in the anterior neuroectoderm; candidate signaling molecules are Cer-1 (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998), Nodal (Varlet et al., 1997), and Dickkopf-1 (Glinka et al., 1998), all of which may antagonize BMP and Wnt signalling (Glinka et al., 1997; Hsu et al., 1998). The former signalling events may induce Otx2 expression in the neuroectoderm, which would then function to maintain expression of Six3, Fkh2, Hesx1 and Pax2 in the rostral territory. Timely, albeit weak, expression of Enl and Wnt1 at 8.5 dpc in the Otx2+/+Otx1-/+ neuroectoderm also suggests independent induction of these genes but maintenance by Otx2. In Drosophila brain en and wg are targets of otd (Cohen and Jurgens, 1991). The knock-in mutation described here suggests that Otx2 function in the early neuroectoderm prior to the onset of Otx1 expression cannot be replaced by Otx1.

Otx2+/- and Otx1+/- embryos was replaced with wild-type tissue (Ep+/+Ve+/-; chimera) (Rhinn et al., 1998). In these chimeras, the prechordal plate was formed and anterior neuroectoderm was induced, but the rostral brain was subsequently deleted. Six3 and Pax2 were induced in neuroectoderm at the 0-4 somite stage, but their expression was almost abolished by the 6 to 8 somite stage. Hesx1 expression was not seen in Ep+/+Ve+/- chimeras. These findings support our observations: loss of the Otx2 function in the visceral endoderm is rescued in Otx2+/-Otx1+/- embryos, while loss of Otx2 in the anterior neuroectoderm is not. In Otx2+/-Otx1-/- embryos anterior neuroectoderm is induced but not maintained.

Otx2 function in regionalization of rostral brain

Puelles et al. postulate that there are six prosomers anterior to the midbrain: p1-p6 (Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Recent observations suggest that regionalization in the brain takes place first in three compartments: the region anterior to zona limitans, which generates p3-p6 prosomers; the region between the zona limitans and the isthmus, which generates p1-p2 and the midbrain; and the rhombomeric region posterior to the isthmus. Two organizing centers are postulated at the anterior neural ridge and at the isthmus (Marin and Puelles, 1994; Crossley and Martin, 1995; Crossley et al., 1996; Joyner et al., 1996; Shimamura and Rubenstein, 1997; Houart et al., 1998). The zona limitans, which expresses the signaling molecule SHH (Echelard et al., 1993) and divides the forebrain into two regions, one competent to respond to signals from the isthmus or Fgf8 (p1, p2) and the other not (p3-p6) (Meinhardt, 1983; Martinetz et al., 1991; Figdor and Stern, 1993; Marin and Puelles, 1994; Rubenstein et al., 1994; Bally-Cuif and Wassef, 1995; Crossley et al., 1996), might be another organizing center. It is still a matter of debate, however, whether and how the rostral brain is developmentally compartmentalized.

At stages later than 8.5 dpc, Otx2+/-Otx1+/- defects were milder than those seen in the Ep+/+Ve+/- chimeras. In the latter, at the 6 to 8 somite stage En and Pax2 expressions were confined to a very small domain at the rostralmost tip of the chimera. At stage 8, En and Pax2 expression was barely detectable, and the posterior of the rostralbrain was subsequently deleted. In Otx2+/-Otx1-/- embryos the isthmus and r1/r2 were formed. At 8.5 dpc some structures existed anterior to the Fgf8- and Pax2-positive isthmus, as evidenced by residual expression of Emx2, Wnt1 and Enl, as well as knocked-in Otx1. Expression of Six3 and Fgf8 in ventral diencephalon and Hesx1 in Rathke’s pouch, though faint, were also detected. The 11.5 dpc knock-in embryos had a cephalic furrow and some rostral brain structures, and some structures developed anterior to the pons and cerebellum in prenatal embryos.

Otx1 is weakly expressed at the 1 somite stage and becomes apparent by the 3 somite stage (Simeone et al., 1999; Suda et al., 1997). In subsequent stages Otx2 may collaborate with Otx1 to regionalize the rostral brain. In contrast to the Ep+/+Ve+/- chimera, Otx2 function at this stage might be replaced by knocked-in Otx1. At present the only means by which this conclusion can be tested is to examine Otx1+/- and Otx2+/- double heterozygotes.

We reported previously that Otx1+/-Otx2+/- brain defects
start around the 3 to 6 somite stage; neither Otx1+/+Otx2+/− nor Otx1−/−Otx2+/− showed obvious defects (Suda et al., 1996, 1997). At the 6 somite stage Otx2 expression in Otx1+/+Otx2+/− embryos was less distinct caudally in the future midbrain region (Suda et al., 1997). Thus Otx2 expression in forebrain and midbrain appears to be independently regulated, and its expression in midbrain may be autoregulated or regulated by the Otx1 gene product in a dose-dependent manner. In the midbrain of Otx1+/−Otx2+/− mutants Wnt1 expression was decreased and Fgf8 expression remained expanded rather than restricted to the future isthmus (Suda et al., 1997). Double heterozygote defects were pronounced in the mesencephalon and caudal diencephalon, and telencephalon and anterior diencephalon were less affected. These defects were mostly restricted to the future isthmus (Suda et al., 1997). Double heterozygotes include dorsal and Otx2−/− embryos, suggesting that Otx2 function at this stage is indeed substitutable by Otx1. Recently, Acampora et al. (1998) demonstrated that Otx1 function at this stage can be substituted for by otd, the Drosophila orthologue, in a dose-dependent manner; the rescue was less efficient in caudal midbrain. It is important to note that this substitution of Otx2 function by Otx1 is in the presence of one Otx2 allele. It remains to be determined if two Otx2 alleles are replaceable with Otx1.

Establishment of midbrain/hindbrain boundary

The isthmus generates midbrain in a mirror image with an antero-posterior gradient when transplanted in p1 and p2 diencephalon (Marin and Puuelles, 1994). Genes such as Pax2, Pax5, Fgf8, Gbx2, En1, En2 and Wnt1 are expressed in a transverse band in the isthmus, and its organizer activity is mimicked by Fgf8 (Crossley et al., 1996). Initially, the caudal limit of Otx2 expression in anterior neuroectoderm is obscure and overlaps with Pax2 and Fgf8 (Suda et al., 1997). The anterior limit of Gbx2 expression in hindbrain is also obscure (Wassarman et al., 1997). Shortly after, the caudal limit of Otx2 expression and the anterior limit of the Gbx2 expression become distinct, establishing a boundary at which Pax2 and Fgf8 expression is confined (Suda et al., 1997; Wassarman et al., 1997). However, it is not yet clear how this boundary, the isthmus, is established. Whether the isthmus plays a role in development of r1 and r2 is also not clear. The establishment of the isthmus occurs after the onset of Otx1 expression, and Otx2 may collaborate with Otx1 in this process (Suda et al., 1997). Lim1 is not expressed in neuroectoderm nor is Otx2 expressed in r1 or r2. Nevertheless, null mutants in either gene fail to develop r1 and r2 (Acampora et al., 1995; Matsuo et al., 1995; Shalvet and Behringer, 1995; Ang et al., 1996). Defects observed in Otx1 and Otx2 double heterozygotes include dorsal expansion of Fgf8 expression and expansion of r1 (Suda et al., 1997); a similar phenotype was observed in Otx1−/−Otx2+/− mutants in a different genetic background (Acampora et al., 1997).

Pax2 expression was not seen in our Otx2−/− embryos in contrast to recent observations by Rhinn et al. (1998). Pax2 was, however, induced in the Otx2+/−/Otx1−/− mutant, as well as in the Ep−/−/Ve+/− chimeras. Thus we conclude that signals from the visceral endoderm and/or anterior mesendoderm are essential for development of the isthmus and r1/r2. In the Ep−/−/Ve+/− chimeras, however, r1 and r2 were lost, and r3 became the most anterior structure of the embryo by the 12 somite stage (9.0 dpc), while in Otx2+/−/Otx1−/− embryos isthmus and r1/2 were maintained. These findings suggest that Otx function in neuroectoderm is also required to establish and/or maintain the isthmus, and that signals from the isthmus and/or caudal midbrain are essential for maintenance of r1 and r2.

Otx2 functions in cephalic neural crest cells

Cephalic neural crest cells, a new development in vertebrates, are known to have played essential roles in the evolution of the rostral head (Newth, 1956; Northcutt and Gans, 1983; Langille and Hall, 1988). These cells were responsible for development of the jaw upon transition from agnatha to gnathostomes, one of the most important innovations in vertebrate history. They were also utilized to develop the anterior cranium to support the expanding brain beyond the anterior tip of the notochord. The phenotype of Otx2+/− mice revealed the role of Otx2 in cephalic crest cells (Matsuo et al., 1995). Populations of cephalic neural crest cells are present in an extant agnatha, the lamprey (Horigome et al., 1999), and those cells express an Otx cognate (our unpublished result); expression of this cognate was detected in the mesenchyme of the first pharyngeal arch and in mesencephalic crest cells known to give rise to trabecular cartilage (Newth, 1956; Langille and Hall, 1988). Thus the recruitment of new downstream targets is what the Otx cognate accomplished with the transition form agnatha to gnathostomes.

Otocephalic defects in Otx2+/− mice were largely restored by Otx1, and we conclude that Otx2 function in mesencephalic crest cells is substitutable by Otx1. It is, however, not clear why defects are not rescued in all Otx2+/− mice. Possibly, subtle differences exist in cephalic crest expression of the knocked-in Otx1 gene compared to expression of endogenous Otx2. Alternatively, the affinities of Otx1 protein to target DNA sequences or to cofactors specific to those cells might be lower than those of Otx2 protein, and otocephalic defects might be completely restored at higher Otx1 dosage.

Divergence into Otx2 and Otx1

Expression patterns of Amphioxus and ascidian Otx cognates suggest an ancestral role for chordate Otx in patterning the anterior mesendoderm and in the involvement of this tissue in induction of anterior neural regions in the overlying ectoderm (William and Holland, 1998). The visceral endoderm, which with the extraembryonic mesoderm forms the visceral yolk sac for maternofetal exchange of nutrients, is, on the other hand, a mammalian innovation. The visceral endoderm may be homologous to the hypoblast in avian species and reptiles, but no directly comparable structure exists in Xenopus or zebrafish. Nevertheless, Otx2 function in the visceral endoderm may represent a universal function throughout vertebrates as a head organizer component. In avian species, the Otx2 cognate is expressed in the epiblast, the hypoblast, Hensen’s node, the anterior mesendoderm and the anterior neuroectoderm (Bally-Cuif et al., 1995). The same is true in Xenopus (Pannese et al., 1995; Kablar et al., 1996), but in zebrafish it is Otx1-cognates that are expressed at gastrulation in the embryonic shield, the fish organizer, and in the anterior axial mesoderm (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995). In zebrafish anterior neuroectoderm, expression of Otx1 cognates also precedes that of the Otx2 cognate. Xenopus and zebrafish Otx cognates are all expressed maternally, the significance of which is not yet known.
Defects in Drosophila otd mutants are restored by OTX2, the human OTX2 homologue, at a higher frequency than by OTX1 (Leuzinger et al., 1998). Thus it is postulated that the ancestral roles of OTX/OTD genes are preserved more in OTX2 cognates in vertebrates, and that OTX1 evolved new functions required for specific vertebrate developmental programs, which otd could not perform. In the Otx1/odt mutant, for example, otd could not rescue a late Otx1 function in the lateral semicircular canal that does not exist in lamprey (Acampora et al., 1998). In contrast, the present study suggests that Otx2 has evolved a new function in establishment of the rostral neuroectoderm. All other Otx2 functions, in visceral endoderm, anterior mesendoderm, regionalization of anterior neuroectoderm and cephalic neural crest cells, were substitutable by Otx1. Such a function, unique to Otx2, might involve acquisition of new target sequences that Otx1 protein does not recognize or alterations in affinity to target sequences and/or cofactors with which Otx1 protein interacts weakly. Alternatively, Otx2 expression may be autoregulated, a regulatory function that cannot be replaced by Otx1 protein, or Otx1 and Otx2 proteins may have different stabilities.

In the gnathostome lineage, when animals diverged into teleost and tetrapod lineages, the ancestor of the former might have retained Otx1 for head organizer function and used it for the subsequent establishment of the territory of the future forebrain and midbrain; the ancestor of the tetrapod lineage might have retained Otx2 for these functions. Later in this lineage, Otx2 might have evolved new functions required for a specific program to establish the tetrapod-unique anterior neuroectoderm, functions that Otx1 could not perform. Unfortunately, however, no Otx cognates have been identified in hagfish, Petromyzon, cartilaginous fish, lungfish, coelacanths or teleosts other than zebrafish. To gain further insight into Otx cognates and the evolution of the vertebrate rostral brain, it will be important to determine whether lamprey OtxA or the zebrafish Otx1 cognate can substitute for Otx2 function in mice.

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